

TITLE OF THE INVENTION

NUCLEOTIDE SEQUENCES WHICH CODE FOR THE *ccpA2* GENE

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to German Application No. DE 10042053.2 filed August 26, 2000 and German Application No. DE 10123071.0 filed May 11, 2001, the entire contents of both applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The invention provides nucleotide sequences from *Coryneform* bacteria which code for the *ccpA2* gene and a process for the fermentative preparation of amino acids, in particular L-lysine, by attenuation of the *ccpA2* gene. The *ccpA2* gene codes for the CcpA2 protein, which is the catabolite control protein A.

DISCUSSION OF THE BACKGROUND

L-Amino acids, particularly L-lysine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and, most particularly, in animal nutrition.

It is known that amino acids are prepared by fermentation of strains of *Coryneform* bacteria, in particular *Corynebacterium glutamicum*. Because of their great importance, attempts are continuously being made to improve the preparation processes. Improvements to the process may concern measures relating to fermentation, for example, stirring and oxygen supply, or the composition of the nutrient media, such as the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

The output properties of these microorganisms are improved by employing methods of mutagenesis, selection and mutant selection. These methods yield strains that produce amino acids and are resistant to antimetabolites or are auxotrophic for metabolites important for regulation.

For a number of years, methods of recombinant DNA technology have also been used for improving the L-amino acid-producing strains of *Corynebacterium*. However, there remains a critical need for improved methods of producing L-amino acids and thus for the provision of strains of bacteria producing higher amounts of L-amino acids. On a commercial or industrial scale even small improvements in the yield of L-amino acids, or the efficiency of their production, are economically significant. Prior to the present invention, it was not recognized that attenuation of the *ccpA2* gene encoding the catabolite control protein A (CcpA2) would improve L-amino acid yields.

#### SUMMARY OF THE INVENTION

An object of the present invention is to provide novel measures for the improved production of L-amino acids or amino acid, where these amino acids include L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-arginine and the salts (monohydrochloride or sulfate) thereof.

One object of the present invention is providing a novel process for improving the fermentative production of said L-amino acids, particularly L-lysine. Such a process includes enhanced bacteria, preferably enhanced *Coryneform* bacteria, which express attenuated amounts of the CcpA2 catabolite control activity.

Thus, another object of the present invention is providing such a bacterium, which expresses an attenuated amount of CcpA2 catabolite control protein or gene products of the *ccpA2* gene.

Another object of the present invention is providing a bacterium, preferably a *Coryneform* bacterium, which expresses a polypeptide that has an attenuated CcpA2 catabolite control activity.

Another object of the invention is to provide a nucleotide sequence encoding a polypeptide which has CcpA2 catabolite control protein sequence. One embodiment of such a sequence is the nucleotide sequence of SEQ ID NO: 1.

A further object of the invention is a method of making CcpA2 catabolite control protein or an isolated polypeptide having a CcpA2 catabolite control activity, as well as use of such isolated polypeptides in the production of amino acids. One embodiment of such a polypeptide is the polypeptide having the amino acid sequence of SEQ ID NO: 2.

Other objects of the invention include methods of detecting nucleic acid sequences homologous to SEQ ID NO: 1, particularly nucleic acid sequences encoding polypeptides that have CcpA2 catabolite control activity, and methods of making nucleic acids encoding such polypeptides.

5 The above objects highlight certain aspects of the invention. Additional objects, aspects and embodiments of the invention are found in the following detailed description of the invention.

#### BRIEF DESCRIPTION OF THE DRAWING

10 **Figure 1.** Map of the plasmid pCR2.1ccpA2int.

#### DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art of molecular biology.

15 Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods,  
20 and examples are illustrative only and are not intended to be limiting.

Reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques, encompassed by the present invention. See, for example, Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1982) and Sambrook et al., *Molecular Cloning: A*  
25 *Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1989) and the various references cited therein.

The invention provides an isolated polynucleotide from *Corynebacterium* bacteria, containing a polynucleotide sequence coding for the *ccpA2* gene, selected from the group consisting of

- 30 a) polynucleotide that is at least 70% identical to a polynucleotide that codes for a polypeptide containing the amino acid sequence of SEQ ID No. 2,  
b) polynucleotide that codes for a polypeptide containing an amino acid sequence that

- is at least 70% identical to the amino acid sequence of SEQ ID No. 2,
- c) polynucleotide that is complementary to the polynucleotides of a) or b), and
  - d) polynucleotide containing at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

5 the polypeptide preferably having the activity of the catabolite control protein CcpA2.

The invention also provides the above-mentioned polynucleotide, preferably being a replicatable DNA containing:

- (i) the nucleotide sequence shown in SEQ ID No.1 or
- (ii) at least one sequence that corresponds to sequence (i) within the range of the  
10 degeneracy of the genetic code, or
- (iii) at least one sequence that hybridizes with the sequences that are complementary to sequences (i) or (ii), and optionally
- (iv) sense mutations in (i) that are neutral in terms of function.

The invention also provides:

15 a replicatable DNA containing the nucleotide sequence as shown in SEQ ID No.1;  
a polynucleotide that codes for a polypeptide containing the amino acid sequence as shown in SEQ ID No. 2;

a vector containing parts of the polynucleotide according to the invention, but at least  
20 15 successive nucleotides (point d, *supra*), particularly pCR2.1ccpA2int,  
deposited in *Escherichia coli* DSM 14257 at the DSMZ, Braunschweig  
(Germany);

and *Coryneform* bacteria that contain in the *ccpA2* gene an insertion or deletion,  
particularly using the vector pCR2.1ccpA2int.

The invention also provides polynucleotides consisting substantially of a  
25 polynucleotide sequence, which are obtainable by screening, by means of hybridization, of a  
corresponding *Coryneform* gene library that contains the complete gene having the  
polynucleotide sequence according to SEQ ID No.1, using a probe containing the sequence of  
said polynucleotide according to SEQ ID No.1 or a fragment thereof, and isolating said  
polynucleotide sequence.

30 Polynucleotide sequences according to the invention are suitable as hybridization  
probes for RNA, cDNA and DNA, in order to isolate the full length nucleic acids or  
polynucleotides or genes that code for the CcpA2 protein, or in order to isolate nucleic acids

or polynucleotides or genes that have a high similarity with the sequence of the *ccpA2* gene.

The DNA of genes that code for the CcpA2 protein can be prepared with the polymerase chain reaction (PCR) by using the polynucleotide sequences according to the invention as primers.

5 Such oligonucleotides acting as probes or primers contain at least 30, preferably at least 20, more preferably at least 15, consecutive nucleotides. Also suitable are oligonucleotides that have a length of at least 40 or 50 nucleotides.

"Isolated" means removed from its natural environment.

10 "Polynucleotide" generally refers to polyribonucleotides and polydeoxyribonucleotides. The RNA or DNA may be modified or un-modified.

The polynucleotides according to the invention include a polynucleotide shown in SEQ ID No. 1 or a fragment prepared therefrom and also those that are at least 70%, preferably at least 80% and in particular at least 90% to 95% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

15 "Polypeptides" are understood as being peptides or proteins that comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide shown in SEQ ID No. 2, particularly those having the biological activity of the CcpA2 protein, and also those that are at least 70%, preferably at least 80% and in particular at least 90% to 95% identical with the polypeptide shown in SEQ ID No. 2 and exhibit the mentioned activity.

20 The invention also provides a process for the production of amino acids, particularly L-lysine, by fermentation using *Coryneform* bacteria which, in particular, already produce amino acids and in which the nucleotide sequences coding for the *ccpA2* gene are attenuated, in particular excluded or expressed at a low level.

25 The term "attenuation" in this connection describes the reduction or exclusion of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, by, for example, using a weak promoter or using a gene or allele that codes for a corresponding enzyme with a low activity, or by inactivating the corresponding gene or enzyme (protein), and optionally by combining those measures. As a result of attenuation, the activity or concentration of the corresponding protein is, in general, reduced to 0 to 50%, 0 to 25%, 0 to 10%, or 0 to 5% of the wild-type protein activity or concentration.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes in a microorganism which are coded by the corresponding DNA, by, for example, increasing the number of copies of the gene or genes, using a potent promoter or using a gene which codes for a corresponding enzyme having a high activity, and optionally combining those measures. As a result of enhancement, in particular over-expression, the activity or concentration of the corresponding protein is increased, in general, preferably ranging from at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400%, or 500%, up to 1000% or 2000% of the wild-type protein activity or concentration present in the microorganism.

The microorganisms provided by the present invention can prepare amino acids, in particular L-lysine, from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. The microorganisms can be representatives of *Coryneform* bacteria, in particular of the genus *Corynebacterium*. *Corynebacterium glutamicum* species of this genus garners special mention since it is well known to those skilled in the art for its ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, particularly the species *Corynebacterium glutamicum* (*C. glutamicum*), are, in particular, the known wild-type strains

*Corynebacterium glutamicum* ATCC13032

*Corynebacterium acetoglutamicum* ATCC15806

*Corynebacterium acetoacidophilum* ATCC13870

*Corynebacterium melassecola* ATCC17965,

*Corynebacterium thermoaminogenes* FERM BP-1539

*Brevibacterium flavum* ATCC14067

*Brevibacterium lactofermentum* ATCC13869 and

*Brevibacterium divaricatum* ATCC14020

or L-amino acid-producing mutants or strains prepared therefrom, for example, the L-lysine-producing strains

*Corynebacterium glutamicum* FERM-P 1709

*Corynebacterium glutamicum* FERM-P 6463

*Corynebacterium glutamicum* FERM-P 6464

*Corynebacterium glutamicum* DM58-1

*Corynebacterium glutamicum* DG52-5

*Corynebacterium glutamicum* DSM 5715

*Corynebacterium glutamicum* DSM 12866

*Brevibacterium flavum* FERM-P 1708 and

*Brevibacterium lactofermentum* FERM-P 1712

5 Preferably, a bacterial strain with attenuated expression of a *ccpA2* gene that encodes a polypeptide with CcpA2 activity will improve amino acid yield at least 1%.

The inventors have succeeded in isolating the new *ccpA2* gene of *C. glutamicum* that codes for the CcpA2 protein, which is a catabolite control protein A.

To isolate the *ccpA2* gene or also other genes of *C. glutamicum*, a gene library of that  
10 microorganism is first prepared in *Escherichia coli* (*E. coli*). The preparation of gene libraries is described in generally known textbooks and handbooks. For example, the textbook of Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990) or the handbook by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989). A well-known  
15 gene library is that of the *E. coli* K-12 strain W3110, which has been prepared by Kohara et al. (Cell 50, 495 -508 (1987)) in  $\lambda$  vectors. Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of *C. glutamicum* ATCC13032, which was prepared with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences, USA, 84:2160-2164) in the *E. coli* K-12 strain NM554  
20 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575). Börmann et al. (Molecular Microbiology 6(3), 317-326) (1992)) in turn describe a gene library of *C. glutamicum* ATCC13032 using the cosmid pH79 (Hohn and Collins, 1980, Gene 11, 291-298).

It is possible to use plasmids such as pBR322 (Bolivar, 1979, Life Sciences, 25, 807-818) or pUC9 (Vieira et al., 1982, Gene, 19:259-268) in order to prepare a gene library of *C.*  
25 *glutamicum* in *E. coli*. Suitable hosts are particularly those *E. coli* strains, which are restriction- and recombination-deficient, such as, the DH5 $\alpha$  strain (Jeffrey H. Miller: "A Short Course in Bacterial Genetics, A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria", Cold Spring Harbour Laboratory Press, 1992).

The long DNA fragments cloned with the aid of cosmids or other  $\lambda$  vectors can then  
30 be subcloned in turn into the usual vectors suitable for DNA sequencing.

Methods of DNA sequencing are described *inter alia* by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America USA, 74:5463-5467,

(1977).

The resulting DNA sequences can then be studied using known algorithms or sequence analysis programs, such as that of Staden (Nucleic Acids Research 14, 217-232 (1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

In that manner, the novel DNA sequence of *C. glutamicum* which codes for the *ccpA2* gene (SEQ ID No. 1) has been obtained and forms part of this invention. Furthermore, the amino acid sequence of the corresponding protein has been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the *ccpA2* gene product is shown in SEQ ID No. 2.

Coding DNA sequences that result from SEQ ID No. 1 by the degeneracy of the genetic code also form part of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 form part of the invention. Furthermore, to a person skilled in the art, conservative amino acid exchanges, such as exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are known as "sense mutations." These mutations do not lead to a fundamental change in the activity of the protein, i.e. are neutral in terms of function. It is also known that changes at the N and/or C terminus of a protein may not substantially impair or may even stabilize the function thereof. The person skilled in the art will find relevant information *inter alia* in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences that result in a corresponding manner from SEQ ID No. 2 also form part of the invention.

Finally, DNA sequences, which are prepared by the polymerase chain reaction (PCR) using primers that result from SEQ ID No. 1 form part of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

A person skilled in the art will find instructions for identifying DNA sequences by means of hybridization *inter alia* in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology 41: 255-260 (1991)). Hybridization takes place under stringent conditions, that is to say only hybrids in which the probe and



target sequence, i.e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. For reasons explained *infra*, the hybridization reaction is preferably carried out under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

A 5x SSC buffer at a temperature of approximately 50 - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with polynucleotides which are less than 70% identical to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995) with a temperature of approximately 50 - 68°C being established. It is optionally possible to lower the salt concentration to 0.1x SSC. Polynucleotide fragments which are, for example, at least 70% or at least 80% or at least 90% to 95% identical to the sequence of the probe employed can be isolated by increasing the hybridization temperature stepwise in approximately 1 - 2°C increments. Commercial kits containing further instructions on hybridization are readily obtainable (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558).

A person skilled in the art will find instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) *inter alia* in the handbook by Gait: Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

During work on the present invention it was found that *Coryneform* bacteria produce amino acids, in particular L-lysine, in an improved manner after attenuation of the *ccpA2* gene.

To achieve an attenuation, either the expression of the *ccpA2* gene or the catalytic properties of the enzyme protein may be diminished or excluded. The two measures may optionally be combined.

The reduction of gene expression may be effected by carrying out the culturing in a suitable manner or by genetic modification (mutation) of the signal structures of gene expression. Signal structures of gene expression are, for example, repressor genes, activator

genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. The person skilled in the art will find information on this in the patent application WO 96/15246, in Boyd and Murphy (Journal of Bacteriology 170: 5949 (1988)), in Voskuil and Chambliss (Nucleic Acids Research 26: 3548 (1998)), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191 (1998)), in Pátek et al. (Microbiology 142: 1297 (1996)), Vasicova et al. (Journal of Bacteriology 181: 6188 (1999)) and in known textbooks of genetics and molecular biology, such as the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations that lead to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art; examples that may be mentioned are the works by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Sugimoto et al. (Bioscience Biotechnology and Biochemistry 61: 1760-1762 (1997)) and Möckel ("Die Threonindehydratase aus *Corynebacterium glutamicum*: Aufhebung der allosterischen Regulation und Struktur des Enzyms", Reports from the Jülich Research Centre, Jül-2906, ISSN09442952, Jülich, Germany, 1994). Summaries are found in known textbooks of genetics and molecular biology, such as that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

These mutations may be transitions, transversions, insertions and deletions. Depending on the effect of the amino acid exchange on the enzyme activity, "missense mutations" or "nonsense mutations" are referred to. Insertions or deletions of at least one base pair (bp) in a gene lead to frame shift mutations, as a consequence incorrect amino acids are incorporated or translation is interrupted prematurely. Deletions of several codons typically lead to a complete loss of the enzyme activity. Instructions on the production of such mutations are part of the prior art and can be found in known textbooks of genetics and molecular biology, such as the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

A common method of mutating genes of *C. glutamicum* is the method of gene disruption and gene replacement described by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)).

In the gene disruption method, a central part of the coding region of the gene of interest is cloned in a plasmid vector that is able to replicate in a host (typically *E. coli*), but not in *C. glutamicum*. Suitable vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pK18mobsacB or pK19mobsacB (Jäger et al., Journal of Bacteriology 174: 5462-65 (1992)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman Journal of Biological Chemistry 269:32678-84 (1994); US Patent 5,487,993), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)) or pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516). The plasmid vector containing the central part of the coding region of the gene is then transferred into the desired strain of *C. glutamicum* by conjugation or transformation. The method for conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a cross-over event, the coding region of the gene in question is interrupted by the vector sequence and two incomplete alleles are obtained, one lacking the 3' end and one lacking the 5' end. This method has been used by Fitzpatrick et al. (Applied Microbiology and Biotechnology 42, 575-580 (1994)) to exclude the *recA* gene of *C. glutamicum*.

In the gene replacement method, a mutation, such as a deletion, insertion or base exchange, is established *in vitro* in the gene of interest. The allele prepared is in turn cloned in a vector that is not replicated in *C. glutamicum* and this is then transferred into the desired host of *C. glutamicum* by transformation or conjugation. After homologous recombination by means of a first cross-over event effecting integration and by means of a suitable second cross-over event effecting an excision in the target gene or in the target sequence, incorporation of the mutation or of the allele is achieved. This method was used by Peters-Wendisch et al. (Microbiology 144, 915 – 927 (1998)) to exclude the *pyc* gene of *C. glutamicum* by a deletion event.

A deletion, insertion or a base exchange can be incorporated into the *ccpA2* gene in this manner.

In addition, it may be advantageous for the production of L-amino acids, in particular

L-lysine, in addition to attenuation of the *ccpA2* gene, to amplify, in particular to over-express, one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid cycle, of the pentose phosphate cycle or of amino acid export and optionally regulatory proteins.

Thus, for example, the preparation of L-lysine, one or more of the genes chosen from the group

- the *lysC* gene which codes for a feed-back resistant aspartate kinase (Accession No.P26512; EP-B-0387527; EP-A-0699759),
- the *dapA* gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),
- the *eno* gene which codes for enolase (DE: 19947791.4),
- the *zwf* gene which codes for the *zwf* gene product (JP-A-09224661),
- the *dapD* gene which codes for tetrahydrodipicolinate succinylase (Wehrmann et al., Journal of Bacteriology 180, 3159-3165 (1998)),
- the *dapE* gene which codes for succinyldiaminopimelate desuccinylase (Wehrmann et al., Journal of Bacteriology 177: 5991-5993 (1995)),
- the *gap* gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992). Journal of Bacteriology 174:6076-6086),
- the *pyc* gene which codes for pyruvate carboxylase (Peters-Wendisch et al.(Microbiology 144, 915 - 927 (1998))
- the *mgo* gene which codes for malate:quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- the *zwa1* gene which codes for the Zwa1 protein (DE: 19959328.0, DSM 13115)
- the *lysE* gene which codes for lysine export (DE-A-195 48 222)

may at the same time be enhanced, in particular over-expressed.

It may also be advantageous for the production of amino acids, in particular L-lysine, in addition to the attenuation of the *ccpA2* gene, at the same time to attenuate one or more of the genes chosen from the group

- the *pck* gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1, DSM 13047),
- the *pgi* gene which codes for glucose 6-phosphate isomerase(US 09/396,478, DSM 12969),
- the *poxB* gene which codes for pyruvate oxidase (DE:1995 1975.7, DSM 13114),
- the *zwa2* gene which codes for the Zwa2 protein (DE: 19959327.2, DSM 13113).

It may also be advantageous for the production of amino acids, particularly L-lysine, in addition to attenuation of the *ccpA2* gene to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

5 The microorganisms prepared according to the invention, for the purpose of production of L-amino acids, in particular L-lysine, can be cultured by batch process (continuous or discontinuous), fed batch, or repeated fed batch process. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the  
10 textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/ Wiesbaden, 1994)).

A suitable culture medium must be used to meet the requirements of the particular strains. Descriptions of culture media for various microorganisms are found in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology  
15 (Washington D.C., USA, 1981). Sugars and carbohydrates, (e.g., glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose), oils and fats, (e.g., soya oil, sunflower oil, groundnut oil and coconut fat), fatty acids (e.g., palmitic acid, stearic acid and linoleic acid), alcohols (e.g., glycerol and ethanol), and organic acids (e.g., acetic acid) may be used as the carbon source. These substances may be used individually or as a mixture.

20 Organic nitrogen-containing compounds (e.g., peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea) or inorganic compounds (e.g., ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate) may be used as the nitrogen source. These substances may be used individually or as a mixture.

25 The phosphorus source may be phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate (or the corresponding sodium-containing salts). Furthermore, the culture medium must comprise salts of metals (e.g., magnesium sulfate or iron sulfate), which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, may be used in addition to the above-mentioned substances.  
30 Moreover, suitable precursors may be added to the culture medium. The starting substances mentioned may be added to the culture in the form of a single batch, or may be added in a suitable manner during fermentation.

Basic compounds (e.g., sodium hydroxide, potassium hydroxide, ammonia or aqueous

ammonia) or acid compounds (e.g., phosphoric acid or sulfuric acid) may be added in a suitable manner to control the pH of the culture. Fatty acid polyglycol esters may be used to control the development of foam. In order to maintain the stability of plasmids, suitable substances having a selective action, such as antibiotics, may be added to the medium. In order to maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as air, are introduced into the culture. The temperature of the culture is normally from 20°C to 45°C, and preferably 25°C to 40°C. Fermentation is continued until the maximum of the desired product has formed. This objective is normally reached within 10 hours to 160 hours.

Methods for the determination of L-amino acids are known from the prior art. The analysis can thus be carried out, for example, by anion exchange chromatography with subsequent ninhydrin derivatization as described by Spackman et al. (Analytical Chemistry, 30, 1190 (1958)) or it can be carried out by reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

The process according to the invention is used for the production of amino acids, in particular L-lysine, by fermentation.

The amino acids are in general isolated by conventional processes or separated off together with constituents of the fermentation broth and optionally the entire biomass or portions thereof.

The isolation of plasmid DNA from *Escherichia coli* and all techniques of restriction, Klenow and alkaline phosphatase treatment were performed as described in Sambrook et al. (Molecular Cloning. A Laboratory Manual, 1989, Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY, USA). Methods for transformation of *Escherichia coli* and the composition of the usual nutrient media, such as LB or TY medium, are also described in this handbook.

The following microorganism was deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

- *Escherichia coli* Top10/pCR2.1ccpA2int as DSM 14257.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

## EXAMPLES

### Example 1

Preparation of a genomic cosmid gene library from *C. glutamicum* ATCC 13032

Chromosomal DNA from *C. glutamicum* ATCC 13032 was isolated as described by  
5 Tauch et al. (1995, Plasmid 33:168-179) and partly cleaved with the restriction enzyme  
*Sau*3AI (Amersham Pharmacia, Freiburg, Germany, Product Description *Sau*3AI, Code no.  
27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase  
(Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Code no.  
1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987), Proceedings of the  
10 National Academy of Sciences, USA 84:2160-2164), obtained from Stratagene (La Jolla,  
USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved  
with the restriction enzyme *Xba*I (Amersham Pharmacia, Freiburg, Germany, Product  
Description *Xba*I, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline  
phosphatase.

15 The cosmid DNA was then cleaved with the restriction enzyme *Bam*HI (Amersham  
Pharmacia, Freiburg, Germany, Product Description *Bam*HI, Code no. 27-0868-04). The  
cosmid DNA so treated was mixed with the treated ATCC13032 DNA and the batch was  
treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description  
T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with  
20 the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description  
Gigapack II XL Packing Extract, Code no. 200217).

For infection of the *E. coli* strain NM554 (Raleigh et al. 1988, Nucleic Acid Res.  
16:1563-1575) the cells were taken up in 10 mM MgSO<sub>4</sub> and mixed with an aliquot of the  
phage suspension. The infection and titering of the cosmid library were carried out as  
25 described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring  
Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) containing  
100 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones were  
selected.

### Example 2

30 Isolation and sequencing of the *ccpA2* gene

The cosmid DNA of an individual colony was isolated with the Qiaprep Spin  
Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the

manufacturer's instructions and partly cleaved with the restriction enzyme *Sau3AI* (Amersham Pharmacia, Freiburg, Germany, Product Description *Sau3AI*, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments ranging from 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, Product Description Zero Background Cloning Kit, Product No. K2500-01) was cleaved with the restriction enzyme *Bam*HI (Amersham Pharmacia, Freiburg, Germany, Product Description *Bam*HI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the *E. coli* strain DH5 $\alpha$ MCR (Grant, 1990, Proceedings of the National Academy of Sciences, U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l zeocin.

Plasmid preparation of the recombinant clones was carried out with the Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). DNA sequencing was administered by the dideoxy chain termination methodology of Sanger et al. (1977, Proceedings of the National Academies of Sciences, U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. Separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZero1 derivatives were assembled to a continuous contig. The computer-assisted coding region analyses were prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231). Further analyses were carried out with the "BLAST search program"



(Altschul et al., 1997, Nucleic Acids Research, 25:3389-3402) against the non-redundant databank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA).

The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of this nucleotide sequence revealed an open reading frame of 1041 bp, which was designated the *ccpA2* gene. The *ccpA2* gene codes for a polypeptide of 346 amino acids (SEQ ID No. 2).

### Example 3

Preparation of an integration vector for integration mutagenesis of the *ccpA2* gene

From the *C. glutamicum* ATCC strain 13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)). On the basis of the sequence of the *ccpA2* gene known for *C. glutamicum* from Example 2, the following oligonucleotides were objectively designed for the polymerase chain reaction:

*ccpA2intA* (SEQ ID NO. 3):

5'AGA GCT GCT TGG TCA GAC TT 3'

*ccpA2intB* (SEQ ID NO. 4):

5'ATC CAG ATT CTT GGC GGT AG 3'

The primers shown were synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press) with Pwo-Polymerase from Boehringer. With the aid of the polymerase chain reaction, an 322 bp internal fragment of the *ccpA2* gene (SEQ ID No. 1) was isolated.

The amplified DNA fragment was ligated into the vector pCR2.1-TOPO (Mead et al. (1991), Bio/Technology 9:657-663) with the TOPO TA Cloning Kit from Invitrogen Corporation (Carlsbad, CA, USA; Catalogue Number K4500-01).

The *E. coli* strain TOP10F was then transformed with the ligation batch (Hanahan, In: DNA cloning. A practical approach. Vol. I, IRL-Press, Oxford, Washington DC, USA 1985). Selection for plasmid-carrying cells was made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2<sup>nd</sup> Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with 25 mg/l kanamycin. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and tested by restriction with the restriction enzyme *EcoRI* and subsequent agarose gel electrophoresis (0.8%). The plasmid was designated

pCR2.1ccpA2int (Figure 1). The abbreviations and designations used in Figure 1 have the following meaning.

KmR:	Kanamycin resistance gene
<i>EcoRI</i> :	Cleavage site of the restriction enzyme <i>EcoRI</i>
<i>HindIII</i> :	Cleavage site of the restriction enzyme <i>HindIII</i>
<i>SacI</i> :	Cleavage site of the restriction enzyme <i>SacI</i>
<i>PstI</i> :	Cleavage site of the restriction enzyme <i>PstI</i>
ccpA2int:	Internal fragment of the <i>ccpA2</i> gene
ColE1 ori:	Replication origin of the plasmid ColE1

#### Example 4

Integration mutagenesis of the *ccpA2* gene in the lysine producer DSM 5715.

The vector pCR2.1ccpA2int mentioned in Example 3 was introduced into *C. glutamicum* DSM 5715 (EP 435 132) by the electroporation method of Tauch et al. (FEMS Microbiological Letters, 123:343-347 (1994)). Strain DSM 5715 is an AEC-resistant lysine producer. The vector pCR2.1ccpA2int is unable to replicate independently in DSM 5715 and is retained in the cell only if it has been integrated into the chromosome of DSM 5715.

Selection of clones with pCR2.1ccpA2int integrated into the chromosome was performed by plating out the electroporation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which had been supplemented with 15 mg/l kanamycin.

For detection of the integration, the *ccpA2int* fragment was labelled with the Dig hybridization kit from Boehringer by the method of "The DIG System Users Guide for Filter Hybridization" of Boehringer Mannheim GmbH (Mannheim, Germany, 1993). Chromosomal DNA of a potential integrant was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 – 1828 (1994)) and in each case cleaved with the restriction enzymes *SalI*, *SacI* and *HindIII*. The fragments formed were separated by agarose gel electrophoresis and hybridized at 68°C with the Dig hybridization kit from Boehringer. The plasmid pCR2.1ccpA2int mentioned in Example 3 had been inserted into the chromosome of DSM 5715 within the chromosomal *ccpA2* gene. The strain was called DSM 5715::pCR2.1ccpA2int.

### Example 5

#### Preparation of L-lysine

The *C. glutamicum* strain DSM 5715::pCR2.1ccpA2int obtained in Example 4 was cultured in a nutrient medium suitable for the production of L-lysine by fermentation, and the L-lysine content in the culture supernatant was determined.

To that end, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with 25 mg/l kanamycin) for 24 hours at 33°C. A pre-culture was inoculated (10 ml medium in a 100 ml conical flask). The complete CgIII medium was used as the medium for the pre-culture starting from this agar plate culture.

#### Cg III Medium

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast extract	10 g/l
Glucose (autoclaved separately)	2% (w/v)
The pH was brought to pH 7.4	

Kanamycin (25 mg/l) was added to the pre-culture medium. The pre-culture was then incubated for 16 hours at 33°C at 240 rpm on a shaker. A main culture was inoculated from this pre-culture so that the initial OD (660 nm) of the main culture was 0.1 OD. MM medium was used for the main culture.

#### MM Medium

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50g/l
Salts:	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	25 g/l
KH <sub>2</sub> PO <sub>4</sub>	0.1 g/l
MgSO <sub>4</sub> * 7 H <sub>2</sub> O	1.0 g/l
CaCl <sub>2</sub> * 2 H <sub>2</sub> O	10 mg/l
FeSO <sub>4</sub> * 7 H <sub>2</sub> O	10 mg/l
MnSO <sub>4</sub> * H <sub>2</sub> O	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
Leucine (sterile-filtered)	0.1 g/l
CaCO <sub>3</sub>	25 g/l

CSL, MOPS and the salt solution are adjusted to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions are then added, as well as the dry, autoclaved  $\text{CaCO}_3$ .

Cell growth was carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) was added. Cell growth was carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of L-lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion-exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	Lysine HCl g/l
DSM 5715	7.9	13.53
DSM 5715::pCR2.1ccpA2int	8.1	14.94

Obviously, numerous modifications and variations on the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.